Journal of Genetic Engineering and Biotechnology (2013) **11**, 129–134

Academy of Scientific Research & Technology and
National Research Center, Egypt

**Journal of Genetic Engineering and Biotechnology**

www.elsevier.com/locate/jgeb

ARTICLE

# Genetic polymorphism detection of two α-Casein genes in three Egyptian sheep breeds

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Received 8 March 2013; revised 19 April 2013; accepted 3 May 2013
Available online 2 June 2013

**KEYWORDS**

Sheep
*αs1-CN*
*αs2-CN*
RFLP
SSCP

**Abstract** Sheep milk is an excellent raw material for the milk processing industry especially in cheese production. The protein content and composition of sheep milk are important in the cheese manufacturing. The casein fraction of ruminant milk proteins consists of four caseins, namely αs1, αs2, β and κ-Casein. Casein genetic polymorphisms are important due to their effects on quantitative traits and technological properties of milk.

This study aimed to detect the genetic polymorphism of αs1- and αs2-Casein genes in three native Egyptian sheep breeds; Rahmani, Barki and Ossimi. PCR-SSCP and PCR-RFLP were used to detect the genetic polymorphism of *αs1-CN* and *αs2-CN* genes, respectively.

A 223-bp fragment of *αs1-CN* gene was amplified by PCR and SSCP results recorded the presence of three different patterns; TT, TC and CC; in 87 tested sheep animals. The sequence analysis of two homologous patterns showed a single nucleotide polymorphism (SNP) (T → C) at position 170. The frequencies of three patterns in the tested sheep breeds were 43.33%, 50.00%, and 6.67% in Rahmani; 83.33%, 13.33%, and 3.33% in Ossimi and 74.07%, 22.22%, and 3.70% in Barki, respectively. Our nucleotide sequences of *αs1-CN* T and C alleles were submitted to GenBank with the accession numbers KF018339 and KF018340, respectively.

The restriction digestion of *αs2-CN* PCR product (1300-bp) by *Tru1*I endonuclease revealed three different genotypes; AA, AG and GG with frequencies of 66.67%, 30.00%, and 3.33% in Rahmani; 96.67%, 3.33%, and 0.00% in Ossimi and 96.15%, 3.85%, and 0.00% in Barki, respectively. The sequence analysis revealed the presence of a single nucleotide polymorphism (A → G) in intron 6 of *αs2-CN* gene. Our nucleotide sequence of *αs2-CN* gene was submitted to GenBank with the accession number JX080380.



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Peer review under responsibility of National Research Center, Egypt.

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## 1. Introduction

The richness of sheep milk and its important value in human health are the main reason for practicing sheep dairying alongside the cow milk industry [18]. The milk processing industry is

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http://dx.doi.org/10.1016/j.jgeb.2013.05.001

focusing on sheep milk as an excellent raw material especially for cheese production [37]. Therefore, it has been suggested that it is better to shift the breeding of sheep from meat and wool to milk [16].

The physicochemical characteristics of sheep milk have unique properties as compared to goat and cow milk. Richness in vitamins A, B and E, calcium, phosphorus, potassium and magnesium is the main characteristic of sheep milk which makes it more nutritious [10], in addition to a higher proportion of short and medium chain fatty acids, which are of known important health benefits [19].

Four caseins, namely αs1, αs2, β and κ-Casein are the main components of the casein fraction of ruminant milk proteins. These four caseins compose 76–86% of total milk protein [36]. The relative amounts of these four casein fractions affect the physicochemical, nutritional and technological properties of ruminant milks [34]. These casein proteins are encoded by four clustered genes in a 250-kb genomic DNA fragment; αs1 is very close to β followed by αs2 and κ-Caseins [24]. These genes were assigned to ovine chromosome 6 (OAR6) [http://www.animalgenome.org/sheep/maps/; 12].

An important step for cheese curd formation is the presence of αs1-Casein (*αs1-CN*) which is also a structural component of the casein micelle [39]. *αs1-CN* constitutes 47.21% of whole ovine milk proteins and is genetically polymorphic due to silent amino acid substitutions or deletions in the triplet code [9,7].

The most highly phosphorylated of calcium sensitive caseins is αs2-Casein (*αs2-CN*), it occurs in sheep milk in several forms and differs on the level of phosphorylation [14]. In bovine, the complete sequence of αs2-Casein gene is comprised of 18 exons ranging in size from 21 to 266 nucleotides, while in ovine it is not known yet [17].

αs2-Casein peptides have unique antibacterial properties [23]. Moreover, αs2-CN peptides with angiotensin-I enzyme inhibitor properties have been identified [38]. On the other hand, αs2-CN peptides could serve to inhibit allergenic responses as suggested by *in vitro* experiments, in addition to the fact that αs2-CN-enriched preparations may have impact in health-promoting or value-added dairy products [22].

This study aimed to detect the genetic polymorphism of αs1- and αs2-Casein genes in three native Egyptian sheep breeds. PCR-SSCP and PCR-RFLP were used to detect the genetic polymorphism of *αs1-CN* and *αs2-CN* genes, respectively.

## 2. Materials and methods

### 2.1. Animals

Whole blood samples were collected from sheep animals belonging to three main sheep breeds reared in Egypt. The blood samples were collected from different farms belonging to Animals Production Institute. The three breeds used in this study are, Rahmani (from Animal Breeding Research Station in Sero, Domiata), Barki (from Animal Breeding Research Station in Borg El-Arab, Alex) and Ossimi (Animal Breeding Research Station in Seds, Bani Swif). The samples were collected from both males and females at different ages.

### 2.2. DNA extraction

Genomic DNA was extracted from the whole blood according to the method described by Miller et al. [26] with minor modifications. Briefly, Blood samples were mixed with cold 2× sucrose-Triton and centrifuged at 5000 rpm for 15 min at 4 °C. The nuclear pellet was suspended in lysis buffer, sodium dodecyl sulfate and proteinase K and incubated overnight in a shaking water bath at 37 °C. Nucleic acids were extracted with saturated NaCl solution. The DNA was picked up and washed in 70% ethanol. The DNA was dissolved in 1× TE buffer. DNA concentration was determined, using Nano Drop1000 Thermo Scientific spectrophotometer, and then diluted to the working concentration of 50 ng/μl, which is suitable for polymerase chain reaction.

### 2.3. Polymerase chain reaction (PCR)

The DNA fragments of the studied genes were amplified through polymerase chain reaction technique developed by Mullis et al. [27]. A PCR cocktail consists of 1.0 μM upper and lower primers (specific for tested genes) (Table 1), 0.2 mM dNTPs and 1.25 U of *Taq* polymerase. The cocktail was aliquoted into PCR tubes with 100 ng of sheep DNA. The reaction was cycled for 35 cycles according to the specific protocol suitable for each primer (Table 1). The amplification was verified by electrophoresis on 2% agarose gel (w/v) in 1× TBE buffer using GeneRuler™ 100-bp ladder as a molecular weight marker for confirmation of the length of the PCR products. The gel was stained with ethidium bromide (1 μg/μl) and visualized on UV trans-illuminator.

### 2.4. Single strand conformation polymorphism (SSCP)

SSCP technique was used to identify the genetic polymorphism of *αs1-CN* gene in 87 animals belonging to the three tested breeds. PCR products were resolved by SSCP analysis according to the method of Orita et al. [28]. PCR product was diluted in denaturing solution, denatured at 94 °C for 5 min, chilled on ice and resolved on polyacrylamide (10%, AA 37:1) with 2% glycerol. The electrophoresis was carried out in a vertical unit in 1× TBE buffer at 200 V and 20 mA for 5 h at 4 °C, the gel

**Table 1** The sequences and information of primers used in this study.

| Gene | Sequences 5′–3′ | PCR conditions 30 cycles | PCR product size | References |
|---|---|---|---|---|
| *αs1-CN* | CAC TGT TGC TTT TTC AAT GGT C<br>AAG GCA ACA ATA TGC AGT CAT TT | 94 °C for 1 min<br>56 °C for 1 min<br>72 °C for 1 min | 223 bp | [5] |
| *αs2-CN* | GCC ATT CAT CCC AGA AAG<br>CTC TTC ATT TGC GTT CCT TA | 94 °C for 45 s<br>54 °C for 45 s<br>72 °C for 2.5 min | 1.3 Kbp | [11] |

was stained with silver staining [2], then visualized on light box and photographed by digital camera.

### 2.5. Restriction fragment length polymorphism (RFLP)

RFLP technique was used to identify the genetic polymorphism of *αs2-CN* gene in 86 animals belonging to the three tested breeds. The restriction mixture was prepared by adding 10 U of the restriction enzyme *Tru1*I to 2.5 μl of restriction buffer and the volume was completed to 5 μl by sterile water. This restriction mixture was mixed with PCR product (20 μl) and incubated at 65 °C for 5 min. The digested PCR products were electrophoresed on a polyacrylamide gel containing ethidium bromide and visualized on UV trans-illuminator.

### 2.6. Sequence analysis

The PCR products of different genotype patterns of *αs1-CN* and *αs2-CN* genes, were purified and sequenced by Macrogen Incorporation (Seoul, South Korea) to identify the SNPs between these different genotype patterns. Sequence analysis and alignments were carried out using CLUSTAL-W [15]. The nucleotide sequences of *αs1-CN* T, *αs1-CN* C and *αs2-CN* in Egyptian sheep were submitted to GenBank (NCBI, BankIt).

## 3. Results and discussion

The four caseins, namely αs1, αs2, β and κ-Casein are the main components of the casein fraction of ruminant milk proteins. These four caseins compose 76–86% of total milk protein [36]. The casein proteins include three main calcium-sensitive proteins which are (αs1-, αs2- and β-Caseins) that coalesce with κ-Casein, calcium and phosphate to form micelles [33].

The effects of casein genetic polymorphisms are important due to their impact on quantitative traits and technological properties of milk [5]. So, caseins have been proposed as polymorphic markers for the selection in order to improve yield and quality of cheese [4]. Globally, the research on polymorphism of ewes' milk protein is not yet as extensive as in cows or goats [20].

### 3.1. αs1-Casein (αs1-CN)

The ovine αs1-Casein gene consists of 19 exons, these exons are small in size, ranging from 24 to 63 nucleotides with the exception of exon 17 and exon 19 which are 155 and 385 nucleotides, respectively, according to the ovine published sequence; GenBank: JN560175.1.

The present study used SSCP technique to study the polymorphism in exon 17 of the αs1-Casein gene in three native Egyptian sheep breeds. A 223-bp fragment was amplified by polymerase chain reaction (Fig. 1). SSCP results recorded the presence of three different patterns; CC, TC and CC; in 87 tested sheep animals (Fig. 2). The sequence analysis of the two homologous patterns showed a single nucleotide polymorphism (SNP) (T → C) at position 170 (Fig. 3). Our nucleotide sequences of *αs1-CN* T and C alleles were submitted to GenBank with the accession numbers KF018339 and KF018340, respectively.

The results showed the presence of pattern III (CC) with low frequency in all tested breeds whereas the pattern I (TT) was found with high frequency. The lowest (3.33%) and highest (83.33%) frequencies were recorded in Ossimi breed for patterns TT and CC, respectively. The frequencies of the three recorded patterns were 4.60%, 28.74% and 66.6% for patterns CC, CT and TT, respectively (Table 2).

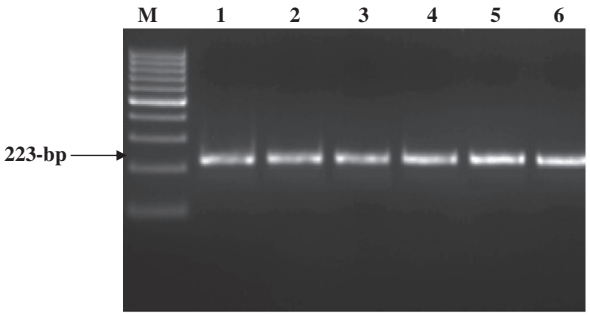


**Figure 1** Agarose gel stained with ethidium bromide showing the PCR product of αs1-Casein gene. M: 100-bp ladder. Lanes 1–6: 223-bp PCR product of αs1-Casein gene.

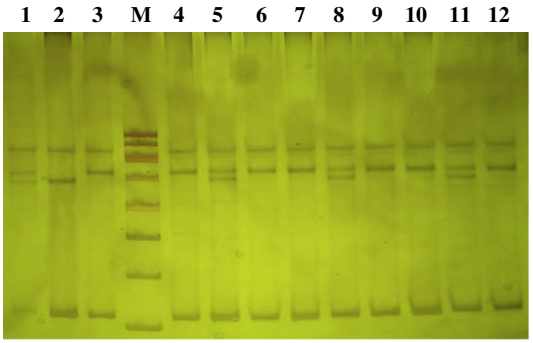


**Figure 2** Three SSCP different patterns of αs1-Casein gene in tested Egyptian sheep on 10% silver stained-polyacrylamide gel. M: 100-bp ladder. Lanes 3, 4, 6, 7, 9, 10 and 12: pattern I (TT). Lanes 1, 5, 8 and 11: pattern II (CT). Lane 2: pattern III (CC).

Ceriotti et al. [5] reported the presence of the same patterns; TT, TC and CC in three Italian sheep breeds under the same conditions used in the present study. These three different patterns resulted from a SNP (T → C) at position 663 which led to the exchange in amino acid $Iso^{186} \rightarrow Thr^{186}$. Later on, Ceriotti et al. [6] examined the presence of the same SNP at exon 17 in five Italian sheep breeds and they recorded that T allele was present at higher frequency than C. The T allele frequency was 0.65 in Gentile di Puglia and Massese, 0.73 in Comisana, 0.81 in Sopravissana and 0.89 in Sarda breed. This result agreed with our result where the frequencies of T and C alleles in the Egyptian sheep were 81.03% and 18.97%, respectively.

Considering the effect of αs1-Casein polymorphism on the milk quality, Chianese [8] found that B variant has been related to milk yield whereas αs1-Casein D variant was related to milk protein percentage [35,1]. Pirisi [32] assessed the effects of αs1-Casein CC, CD and DD genotypes on milk composition and cheese yield. They reported that αs1-Casein CC milk had better cheese making characteristics than DD milk while CD milk had intermediate characteristics. This effect was confirmed by Martini et al. [25] who pointed that; C allele of αs1-Casein is most favorable for cheese making.

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Pattern I   (TT) CACTGTTGCTTTTTCAATGGTCTTTCTCTCTAGCTTTTCAGACAATTCTACCAGCTGGAC 60
Pattern III (CC) ************************************************************ 60

Pattern I   (TT) GCCTATCCATCTGGTGCCTGGTATTACCTTCCACTAGGCACACAATACACTGATGCCCCC 120
Pattern III (CC) ************************************************************ 120

Pattern I   (TT) TCATTCTCTGACATCCCTAATCCCATTGGCTCTGAGAACAGTGGAAAGATTACTATGCCA 180
Pattern III (CC) **************************************************C********** 180

Pattern I   (TT) CTGTGGTGGTAAGTTCATTTAAATGACTGCATATTGTTGCCTT 223
Pattern III (CC) ***************************************** 223
```

**Figure 3** The sequences of two homologous patterns of αs1-Casein gene in Egyptian sheep (alignment by ClustalW2) showed SNP (T → C) at position 170.

**Table 2** The pattern frequencies of the αs1-Casein gene in three tested Egyptian sheep breeds.

| Breeds | No. of animal | Pattern frequencies | | | | | |
|---|---|---|---|---|---|---|---|
| | | Pattern I (TT) | | Pattern II (CT) | | Pattern III (CC) | |
| | | No. of animals | Freq. (%) | No. of animals | Freq. (%) | No. of animals | Freq. (%) |
| Barki | 27 | 20 | 74.07 | 6 | 22.22 | 1 | 3.70 |
| Rahmani | 30 | 13 | 43.33 | 15 | 50.00 | 2 | 6.67 |
| Ossimi | 30 | 25 | 83.33 | 4 | 13.33 | 1 | 3.33 |
| Total | 87 | 58 | 66.67 | 25 | 28.74 | 4 | 4.60 |

### *3.2. αs2-Casein (αs2-CN)*

One of the most highly phosphorylated of calcium sensitive caseins is *αs2-CN*, it occurs in milk in several forms and differs on the level of phosphorylation [14]. The complete sequence of ovine αs2-Casein gene is not known yet, while in bovine; it is comprised of 18 exons ranging in size from 21 to 266 nucleotides [17].

In the present study; PCR-RFLP technique was used to detect the polymorphism within sheep αs2-Casein gene. Polymerase chain reaction amplified a 1300-bp fragment (Fig. 4).

The digestion of the PCR fragments by *Tru1*I endonuclease enabled us to differentiate between three different genotypes; AA, AG and GG. The sizes of digested fragments were not previously recorded, so we used FastPCR program (http://primerdigital.com/fastpcr.html) on the sequences of PCR products of the two different alleles A and G to detect the restriction sites and determine the fragment sizes. The restriction site for *Tru1*I enzyme was found to be (TˆTAA) and the results of *in silico* RFLP showed that the two alleles A and G have four common fragments with sizes of 268-, 213-, 109- and 98-bp (Fig. 5), in addition to some small fragments (they did not show in the figure).

Allele G has a specific fragment at size of 196-bp whereas allele A has a specific fragment with size of 166-bp and another small fragment at size 30-bp. These specific fragments were detected due to the presence of a single nucleotide polymorphism (G/A) in allele A, at position 600 of the sequenced fragment, which led to the presence of an additional restriction site in this allele (Fig. 6). Our sequence of *αs2-CN* gene was submitted to GenBank with the accession number JX080380.

Table 3 reported the genotype and allele frequencies of αs2-Casein gene. The results showed the predominance of AA genotype in all tested breeds over GG genotype which was completely absent in both Barki and Ossimi breeds. AG genotype was present with different frequencies ranging from 3.33% in Ossimi to 3.85% in Barki and 30.00% in Rahmani

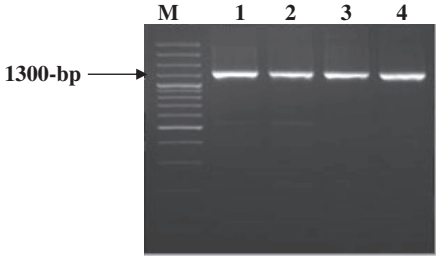


**Figure 4** Agarose gel stained with ethidium bromide showing PCR product of αs2-Casein gene. M: 100-bp plus ladder. Lanes 1–4: 1300-bp PCR product of αs2-Casein gene.

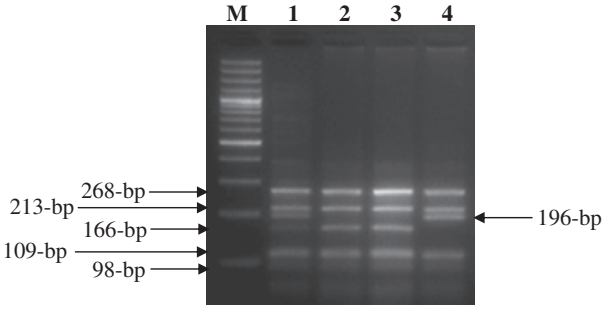


**Figure 5** Agarose gel stained with ethidium bromide showing three different genotypes of αs2-Casein gene after digestion of PCR products with *Tru1*I restriction enzyme. (The small fragments did not show in figure). M: 100-bp plus ladder. Lanes 1: genotype AG. Lanes 2 and 3: genotype AA. Lane 4: genotype GG.

breeds. These genotype frequencies declared the presence of allele A with a high frequency (92.44%) in all tested breeds whereas the allele G showed low frequency (7.56%).

Restriction fragment length polymorphism in ovine *αs2-CN* locus was studied by Di Gregorio et al. [13] and Levéziel et al. [21]. They used *Eco*RV endonuclease digestion of genomic DNA and hybridization with a bovine αs2-Casein cDNA probe and they detected a three-allele polymorphism. On the other hand, Phua et al. [30] observed two allelic *Eco*RI- *αs2-CN* fragments in sheep.

Boisnard et al. [3] reported two genetic variants depending on two amino acid exchanges; $Asn^{49} \rightarrow Asp^{49}$ and $Lys^{200} \rightarrow Asp^{200}$. Recently, Picariello et al. [31] studied the genetic variants at amino acid level in Italian sheep. They observed that B variant differed from the most common variant A with two amino acid exchanges: $Asp^{75} \rightarrow Tyr^{75}$ and $Iso^{105} \rightarrow Val^{105}$. The first one, resulting in a loss of a negative charge, is responsible for the higher isoelectric point of B protein variant which modifies the protein electric charge and maybe effects on milk properties.

The PCR amplified fragments in the present study included intron 5, exon 6 and intron 6 of αs2-Casein gene. *Tru1*I digestion of αs2-Casein amplified fragments differentiated between two alleles A and G. These two alleles have common fragments at sizes of 268, 213, 109, 98, 59, 32, and 30-bp. Allele G has a specific fragment at 196-bp which was further digested into two bands at 166- and 30-bp for allele A (Fig. 5). The sequence analysis of amplified fragments for the two different alleles declared that the difference between these two alleles was due to one SNP (A → G). The site of this SNP is equivalent to nucleotide base No. 7886 in the bovine αs2-Casein gene (GenBank: M94327.1) found in intron 6.

Our results for ovine αs2-Casein genetic polymorphism were different from goat, where Cosenza et al. [11] and

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Allele A  TAACAGAGAGGGGTGGAGGTTTGTCTTCATATTTATGCTATATACTAATGAAAGGAATTC 60
Allele G  ************************************************************ 60

Allele A  TGGGAAAAGATTTGAGAGCCATTTTTGAGCCACAATATTAAAATAGGATATCTTTGAAAG 120
Allele G  ************************************************************ 120

Allele A  AATTTCTTGTTAAAGATTCATCATTTTTACTTTTCTTCCTTAATTCTGTACTCTTTTGTT 180
Allele G  ************************************************************ 180

Allele A  CACAAAATCATATTTTCTTTGCTATAACTATATCCTCAAGAATATTATACTACATTTCAG 240
Allele G  ************************************************************ 240

Allele A  TAAATGTTATATCCTTTTGGCTTTGTTTTCTTTTAGGAGAAACTTTGCACCACATCCTGT 300
Allele G  ************************************************************ 300

Allele A  GAGGTACACACTGATTTCACATCTATAGCATAATGTGAAGTAAAATATTATAGTATTTGA 360
Allele G  ************************************************************ 360

Allele A  ACTACACTGAAATTAACCTTTATTTGGAAAATATATTTCAAATCAATAAAACTTGAAAAC 420
Allele G  ************************************************************ 420

Allele A  CAAGATTATCTTTAAACAATGAGACCAAAATTATAGTCTTCTCAATTCTATTACTAAATC 480
Allele G  ************************************************************ 480

Allele A  AAATGAAGAAAATATATCTTTGCATGAATCAACTAACACATTTGATTCCTAGAATCTATG 540
Allele G  ************************************************************ 540

Allele A  AAGAATGAAGAACTTATCTTTTAGTGTTTTTCAGTATCAAACTTATTTTATTCTTTTTTA 600
Allele G  ************************************************************G 600

Allele A  AAATTCTTTTTCTGTTACAATATTTTCTTAAAACTGACTCAATCAATTTATCACAAAAAC 660
Allele G  ************************************************************ 660

Allele A  AATATATATTTGCTTCCTAAAGCATGTCAGTTGCCCTAGTGATGTATTTTTCCTAATACA 720
Allele G  ************************************************************ 720

Allele A  TAATAAGTAACGACTATTAAAATGAGAAAGAAAAATCTATCCATGCCTTGCCTAAAGTTA 780
Allele G  ************************************************************ 780

Allele A  TCTTGTATATTCATTCATTCACTTGACAAATCTTTTATTCAACAAGAACTATGTCCCCAA 840
Allele G  ************************************************************ 840

Allele A  CATTGTCTTAGTCTCTAGGAATACAGTAATAATTATGAGACTCATTGGGGATACAACAAT 300
Allele G  ************************************************************ 900

Allele A  AAACACAGTAAACAACAATAATTACCGCTGTCATCAAATAGGAAATTCTGTTATAACTTA 960
Allele G  ************************************************************ 960

Allele A  TACAAAACTCATACAATGAAAGCGAATCCAGTCTAATGCATCTCT 1005
Allele G  ******************************************** 1005
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**Figure 6** A part of PCR product sequences of two different alleles in Egyptian sheep αs2-Casein gene showing the SNP (G/A) at position 600 of the sequenced fragment (alignment by ClustalW2).

**Table 3** Genotype and allele frequencies of the αs2-Casein gene in three tested Egyptian sheep breeds.

| Breed | No. of animals | Genotype frequencies | | | | | | Allele frequencies | |
|---|---|---|---|---|---|---|---|---|---|
| | | AA | | GG | | AG | | A | G |
| | | No. of animals | Freq. (%) | No. of animals | Freq. (%) | No. of animals | Freq. (%) | Freq. (%) | Freq. % |
| Barki | 26 | 25 | 96.15 | 0 | 0.00 | 1 | 3.85 | 98.08 | 1.92 |
| Rahmani | 30 | 20 | 66.67 | 1 | 3.33 | 9 | 30.00 | 81.67 | 18.33 |
| Ossimi | 30 | 29 | 96.67 | 0 | 0.00 | 1 | 3.33 | 98.33 | 1.67 |
| Total | 86 | 74 | 86.05 | 1 | 1.16 | 11 | 12.79 | 92.44 | 7.56 |

Othman and Ahmed [29] used the same primer to amplify goat αs2-Casein gene and digested the amplified fragments with *Mse*I endonuclease which had the same restriction site of *Tru1*I (TˆTAA). They detected two different alleles with common fragments at sizes of 270- and 230-bp. The allele A had a specific band at 300-bp whereas the specific band for allele B was at 400-bp.

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